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Økonomi- og Erhvervsministeriet

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Pia Høybye-Olsen

PATENT- OG VAREMÆRKESTYRELSEN

METHOD FOR MAKING RECOMBINANT PROTEINS

Field of the Invention

The present invention relates to scaffold/matrix attachment regions isolated from hamster cells and their use for the industrial production of recombinant proteins.

Background of the Invention

Advances in cell culture and recombinant DNA technologies have facilitated the expression of a variety of proteins of therapeutic or other economic value using genetically engineered cells. The expression of many biologically active therapeutic proteins, which are derived from higher eukaryotic sources, often requires specific post-translational modifications which do not naturally occur in lower eukaryotic or prokaryotic cells, thus necessitating the use of cells derived from higher eukaryotic sources. For example, the expression of glycoproteins in mammalian cells has the advantage of providing proteins which contain natural glycosylation. Mammalian-produced glycoproteins contain outer chain carbohydrate moieties which are markedly different from the outer chain carbohydrate moieties present on glycoproteins produced from lower eukaryotes. The use of mammalian cells as hosts for the production of secreted mammalian proteins has the significant advantage over secretion from lower eukaryotes in that mammalian cells have a secretory system that readily recognizes and properly processes secretion-directed proteins, which is not necessarily true for lower eukaryotes.

Scaffold Attachment Regions (SAR), also called Matrix Attachment Regions (MAR) or Scaffold/Matrix Attachment Regions (S/MAR) are non-consensus-like AT-rich DNA elements several hundred base pairs (bp) in length, which organize the nuclear DNA of the eukaryotic genome into some 60,000 chromatin domains, 4 – 200 kbp loops, by periodic attachment to the protein scaffold or matrix of the cell nucleus. S/MARs have been isolated from regions surrounding actively transcribed genes but also from introns, centromeres and teleomeric regions and have been found to collaborate with enhancers to help regulate transcription by controlling the chromatin state of DNA. The observations that S/MARs positively interact with enhancers, form loop domains and often are located at the borders of transcriptionally active domains have led to the idea of using S/MARs as flanking elements around transgenes, forming so called mini-domains, in order to protect transgenes or expression cassettes from transcriptional silencing and the effects of surrounding heterochromatin (transcriptionally inactive chromatin) as well as possibly increase gene expression. Several publications have shown that S/MARs in a flanking position can strongly stimulate expression of transgenes, as well as reduce expression variability between cell clones (position effects).

Moreover, due to th character of a mini-domain, expression should be independent from the integration site.

US patent No. 5,985.607 discloses the use of certain SAR elements for expression of EPO and tPA . In Biochemistry, 30:1264-1270, 1991 the effect of other SAR elements on expression of the human interferon β is disclosed. Also Phi-Van et al. in Mol. Cell. Biol, 10:2302-2307, 1990 ("The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes") disclose the influence on gene expression of a MAR element located upstream of the chicken lysozyme gene.

The proteins involved in the clotting cascade, including, e.g., Factor VII, Factor VIII, Factor IX, Factor X, and Protein C, are proving to be useful therapeutic agents to treat a variety of pathological conditions. Because of the many disadvantages of using human plasma as a source of pharmaceutical products, it is preferred to produce these proteins in recombinant systems. The clotting proteins, however, are subject to a variety of co- and post-translational modifications, including, e.g., asparagine-linked (N-linked) glycosylation; O-linked glycosylation; and γ-carboxylation of Glu residues. For this reason, it is preferable to produce them in mammalian cells, which are able to modify the recombinant proteins appropriately. Production of recombinant proteins within mammalian cells can be difficult because of a low genetic stability of the recombinant gene and/or silencing of the recombinant gene. Several molecular mechanisms have been reported that may lead to gene silencing, a. o. DNA methylation and histone deacetylation.

Thus, there is a need in the art to overcome the deficiencies of the known methods for making clotting proteins by providing mammalian production strains with a higher genetic stability in large-scale production to produce industrial quantities of the clotting proteins, particularly recombinant human Factor VII or Factor VII-related polypeptides.

Summary of the Invention

The present invention is based on the discovery of new S/MAR elements from hamster-derived CHO and BHK cells, which may be used for increasing and stabilizing the expression yield of recombinant proteins in mammalian cells. The S/MAR elements are thought to increase the genetic stability of nearby transcription cassettes and to inhibit gene silencing tby interfering with mechanisms such as DNA methylation and histone deacetylation. Furthermore, the presence of S/MAR elements is thought to decrease clone-to-clone variability through decreasing position effects, thereby minimalizing the need of screening work to identify high-yielding producer cell clones.

The S/MAR el ments will typically be inserted upstream and downstr am to the DNA encoding the desired protein and will be introduced in the host mammalian cell line on expression vectors comprising the necessary elements for expression of heterologous proteins in transfected cells.

Thus in one embodiment the present invention relates to a method for production of Factor VII polypeptides or Factor VII-related polypeptides comprising a) transfecting a mammalian cell with an expression vector comprising a nucleic acid molecule encoding Factor VII or a Factor VII-related polypeptide, expression control regions operatively linked to thereto and at least one S/MAR element; b) culturing the transfected cell under conditions for expression of Factor VII or a Factor VII-related polypeptide and c) isolating the expressed polypeptide by suitable means.

In one embodiment the vector comprises two S/MAR elements flanking the DNA encoding Factor VII or a Factor VII-related polypeptide and the expression control regions. The S/MAR elements may be selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, functional fragments thereof and sequences being at least 70% homologous thereto.

In a further embodiment the S/MAR elements will be at least 75%; 80%, 85%, 90% or 95% homologous to SEQ ID NO:1 or 2, respectively as determined by pairwise DNA sequence alignment using matching methods like the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul, S. F. et al., 1990, "Basic local alignment search tool". J. Mol. Biol. 215:403-410; and Altschul, S. F. et al., 1997, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". Nucl. Acids Res. 25:3389-3402). Examples of S/MAR sequences homologuos to SEQ ID NO:1 and 2 are SEQ ID NO:3; 4 and 5.

The S/MAR elements may be the same or different and may be located in a distance of from 0 to10 kb from the DNA encoding Factor VII or a Factor VII-related polypeptide and the expression control regions.

In one embodiment of the present invention the vector comprises two S/MAR elements being the same. In a further embodiment the S/MAR elements are the same and are selected from the group consisting of SEQ ID NO:1; 2; 3; 4; and 5.

In a further embodiment the vector comprises two S/MAR elements which are different from each other with respect to base pairs and numbers.

The present invention encompasses any combination of SEQ ID NO:1; 2; 3; 4; and 5.

In another embodiment the present invention is related to a method for production of polypeptides or proteins in transfected mammalian cells comprising a) transfecting a mammalian cell with an expression vector comprising a nucleic acid molecule encoding a polypeptide or protein, expression control regions operatively linked thereto and at least one

S/MAR element selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, functional fragments thereof and sequences being at least 70% homologous thereto; b) culturing the transfected cell under conditions for expression of the desired polypeptides or protein and c) isolating the expressed product by suitable means.

The S/MAR elements will in a further embodiment be at least 75%; 80%, 85%, 90% or 95% homologous to SEQ ID NO:1 or 2, respectively as determined by pairwise DNA sequence alignment using matching methods like the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul, S. F. et al., 1990, "Basic local alignment search tool". J. Mol. Biol. 215:403-410; and Altschul, S. F. et al., 1997, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". Nucl. Acids Res. 25:3389-3402). Examples of S/MAR sequences homologuos to SEQ ID NO:1 and 2 are SEQ ID NO:3; 4 and 5.

The S/MAR elements may be the same or different and may be located in a distance of from 0 to10 kb from the DNA encoding Factor VII or a Factor VII-related polypeptide and the expression control regions.

In one embodiment of the present invention the vector comprises two S/MAR elements being the same and selected from the group consisting of SEQ ID NO:1; 2; 3; 4; and 5.

In a further embodiment the vector comprises two S/MAR elements which are different from each other with respect to base pairs and numbers. In this embodiment, the pair of S/MAR elements can be any combination of SEQ ID NO:1; 2; 3; 4; and 5.

In a further embodiment the present invention is related to an isolated DNA molecule having a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and 2 and sequences being at least 70% homologous thereto or sequences that hybridize to the isolated DNA under stringent conditions.

In a still further embodiment the present invention is related to mammalian cell lines transfected with an expression vector comprising a nucleic acid molecule encoding Factor VII or a Factor VII-related polypeptide, expression control regions operatively linked to thereto and at least one S/MAR element.

In still a further embodiment the present invention is related to mammalian cell lines transfected with an expression vector comprising a nucleic acid molecule encoding a polypeptide or protein expression control regions operatively linked to thereto and at least one S/MAR element selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2 , functional fragments thereof and sequences being at least 70% homologous thereto

Brief Description of the Drawings

The invention is further explained with reference to the drawings in which:

Figure 1 shows plasmid pSEAP2-Hygro-MCS;

Figure 2 shows plasmid pSEAP2-CMVp;

Figure 3 shows plasmid pB4B1-SEAP2-B4B1;

Figure 4 shows plasmid pLRCA2-SEAP2-LRCA2;

Figure 5 shows plasmid delta-LRCA2-SEAP2-LRCA2;

Figure 6 shows plasmid pFVII-CMVp;

Figure 7 shows plasmid delta-LRCA2-FVII-HYG-LRCA2; and

Figure 8 shows plasmid B4B1-FVII-HYG-B4B1

Figure 9 shows SEAP expression data from transfected CHO cells

Detailed Description of the Invention

Definitions

The terms Scaffold Attachment Regions (SAR) or Matrix Attachment Regions (MAR) or Scaffold/Matrix Attachment Regions (S/MAR) as used herein refer to non-consensus-like AT-rich DNA elements several hundred base pairs (bp) in length, which organize the nuclear DNA of the eukaryotic genome into some 60,000 chromatin domains, 4 – 200 kbp loops, by periodic attachment to the protein scaffold or matrix of the cell nucleus. S/MAR elements are typically found in non coding regions such as flanking regions, such as regions upstream or downstream to a coding region, and introns.

The S/MAR elements according to the present inventions were isolated from BHK cells and CHO cells, e.g. CHO cell line DG44 (Urlaub, G. et al., 1983, "Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells". Cell 33:405-412).

Methods for identifying S/MAR elements are computer prediction using MAR-FINDER software to search for several sequence motifs, f. ex. origin of replication, AT-rich sequence, TG-rich sequence, and curved DNA sequence, (Singh, G. B., Kramer, J. A. and Krawetz, S. A. (1997). "Mathematical model to predict regions of chromatin attachment to the nuclear matrix". Nucl. Acids Res. 25:1419-1425).

With at least 70% homology is meant DNA wherein the nucleotide sequence is least 70% homologous to a defined sequence measured by pairwise DNA sequence alignment using matching methods like the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul, S. F. et al., 1990, "Basic local alignment search tool". J. Mol. Biol. 215:403-410; and Altschul, S. F. et al., 1997, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". Nucl. Acids Res. 25:3389-3402).

The term functional fragments of SEQ ID NO:1 and 2 as used herein means fragments of said sequences of a size large enough to have the desired effect on expression yields. The functional fragments will typically contain at least 300 bp of the full size of SEQ ID NO:1 and 2. The fragments will preferably be a consecutive sequence of the original sequence. Examples of such fragments are SEQ ID NO:3 from bp 543 to bp 2545 of SEQ ID NO:1; SEQ ID NO:4 from bp 437 to bp 2715 of SEQ ID NO:1 and SEQ ID NO:5 having a 12 base pair deletion in the GT rich region from bp 1551 to 1604 in SEQ ID NO:1.

The term flanking means that the sequences in question are either directly connected to the expression cassette or are connected by linking DNA sequences which may be up to 10 kb or more in length as long as such linking sequences do not interfere with the desired effect of the S/MAR elements. The expression cassette comprise at a minimum a gene of interest and expression control elements operatively linked thereto. Typically S/MAR elements will flank both sides, 5'-upstream and 3'-downstream, of the expression cassette. The orientation of the S/MAR elements is optional and expression cassette can be flanked by all possible combinations of the S/MAR sequences SEQ ID:1; SEQ NO:2; SEQ ID3; ID NO:4 and SEQ ID NO:5.

The control elements will comprise the usual regulatory elements such as transcriptional promoters, enhancers, RNA polymerase binding sites, polyadenylation sites, translation initiation signals, and termination signals and may be readily accomplished by one of ordinary skill in the art.

By human Factor VII polypeptides is meant polypeptides having the amino acid sequence disclosed in U.S. Patent No. 4,784,950 (wild-type Factor VII). As used herein, "Factor VII" or "Factor VII polypeptide" encompasses wild-type Factor VII, as well as variants of Factor VII exhibiting substantially the same or improved biological activity relative to wild-type Factor VII. The term "Factor VII" is intended to encompass Factor VII polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated Factor VIIa. Typically, Factor VII is cleaved between residues 152 and 153 to yield Factor VIIa.

As used herein, "Factor VII-related polypeptides" encompasses polypeptides, including variants, in which the Factor VIIa biological activity has been substantially modified or reduced relative to the activity of wild-type Factor VIIa. These polypeptides include, without limitation, Factor VII or Factor VIIa that has been chemically modified and Factor VII variants into which specific amino acid sequence alterations have been introduced that modify or disrupt the bioactivity of the polypeptide.

The biological activity of Factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of Factor IX or Factor X to produce activated Factor IX or X (Factor IXa or Xa, respectively). For purposes of the invention, Factor VIIa biological activity may be quantified by measuring the ability of a preparation to promote blood clotting using Factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Patent No. 5,997,864. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "Factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml Factor VII activity. Alternatively, Factor VIIa biological activity may b quantified by (i) measuring the ability of Factor VIIa to produce of Factor Xa in a system comprising TF embedded in a lipid membrane and Factor X. (Persson et al., *J. Biol. Chem.* 272:19919-19924, 1997); (ii) measuring Factor X hydrolysis in an aqueous system (see, Example 5 below); (iii) measuring its physical binding to TF using an instrument based on surface plasmon resonance (Persson, *FEBS Letts.* 413:359-363, 1997) (iv) measuring hydrolysis of a synthetic substrate and (v) measuring generation of thrombin in a TF-independent in vitro system.

Factor VII variants having substantially the same or improved biological activity relative to wild-type Factor VIIa encompass those that exhibit at least about 25%, preferably at least about 50%, more preferably at least about 75% and most preferably at least about 90% of the specific activity of wild-type Factor VIIa that has been produced in the same cell type, when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above. Factor VII variants having substantially reduced biological activity relative to wild-type Factor VIIa are those that exhibit less than about 25%, preferably less than about 10%, more preferably less than about 5% and most preferably less than about 1% of the specific activity of wild-type Factor VIIa that has been produced in the same cell type when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above. Factor VII variants having a substantially modified biological activity relative to wild-type Factor VII include, without limitation, Factor VII variants that exhibit TF-independent Factor X proteolytic activity and those that bind TF but do not cleave Factor X.

Variants of Factor VII, whether exhibiting substantially the same or better bioactivity than wild-type Factor VII, or, alternatively, exhibiting substantially modified or reduced bioactivity relative to wild-type Factor VII, include, without limitation, polypeptides having an amino acid sequence that differs from the sequence of wild-type Factor VII by insertion, deletion, or substitution of one or mor amino acids. Non-limiting examples of Factor VII variants having

substantially the same biological activity as wild-type Factor VII include S52A-FVIIa, S60A-FVIIa (Iino et al., *Arch. Biochem. Biophys.* 352: 182-192, 1998); FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., *Biotechnol. Bioeng.* 48:501-505, 1995); and oxidized forms of Factor VIIa (Kornfelt et al., *Arch. Biochem. Biophys.* 363:43-54, 1999). FVII variants as disclosed in PCT/DK02/00189; and FVII variants exhibiting increased proteolytic stability as disclosed in WO 02/38162 (Scripps Research Institute); FVII variants having a modified Gla-domain and exhibiting an enhanced membrane binding as disclosed in WO 99/20767 (University of Minnesota); and FVII variants as disclosed in WO 01/58935 (Maxygen ApS).

Non-limiting examples of FVII variants having increased biological activity compared to wild-type FVIIa include FVII variants as disclosed in WO 01/83725, WO 02/22776, Danish patent application PA 2001 01413, Danish patent application PA 2001 01627; and FVIIa variants with enhanced activity as disclosed in JP 2001061479 (Chemo-Sero-Therapeutic Res Inst.).

Non-limiting examples of Factor VII variants having substantially reduced or modified biological activity relative to wild-type Factor VII include R152E-FVIIa (Wildgoose et al., *Biochem* 29:3413-3420, 1990), S344A-FVIIa (Kazama et al., *J. Biol. Chem.* 270:66-72, 1995), FFR-FVIIa (Holst et al., *Eur. J. Vasc. Endovasc. Surg.* 15:515-520, 1998), and Factor VIIa lacking the Gla domain, (Nicolaisen et al., *FEBS Letts.* 317:245-249, 1993). Non-limiting examples of chemically modified Factor VII polypeptides and sequence variants are described, e.g., in U.S. Patent No. 5,997,864.

The vector system may comprise two separate vectors being capable of expressing FVII and an endoprotease, respectively. In this embodiment the mammalian cells are co-transfected with the two vectors and then cultured in a suitable culture medium. Alternatively an already established Factor VII expression clone may be transfected with a vector capable of expressing the endoprotease. The vector system may also comprise one single vector comprising the FVII expression cassette and the endoprotease expression cassette. An example of sutable endoproteases is the KEX2 enzyme.

The mammalian cells may include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, HEK 293 cells or other immortalized cell lines available, e.g., from the American Type Culture Collection.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39), HEK 293 (ATCC CRL 1573) or CHO (ATCC CCL 61) cell lines.

Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

The vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The vector is preferably an expression vector in which the encoding DNA sequence is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the encoding DNA in mammalian cells are the SV40 promoter (Subramani, S. et al., 1981, "Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid I simian virus 40 vector", Mol. Cell. Biol. 1:854-864), the MT-1 (metallothionein gene) promoter (Palmiter, R. D. et al., 1983, "Metallothionein-human HG fusion genes stimulate growth of mice". Science 222:809-14) or the human cytomegalovirus immediate-early promoter (Nelson, J. A and Groudine, M., 1986, "Transcriptional regulation of the human cytomegalovirus major immediate-early gene is associated with induction of DNase I hypersensitive sites". Mol. Cell. Biol 6:452-461).

The encoding DNA sequence may also, be operably connected to a suitable terminator, such as the human growth hormone terminator or the ADH3 terminator.

The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the

SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The vector will also preferably contain a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of the FVII polypeptide which can direct the expressed FVII polypeptide into the cell's secretory pathway of the host cell. The signal may be homologous or heterologous to the host mammalian cell line and it may be the natural signal peptide

Finally, the vector may comprise a DNA sequence enabling it to replicate in the host cell in question. An example of such a sequence in a mammalian cell is the SV40 origin of replication.

The transfected mammalian cells are cultured in a suitable nutrient medium under conditions permitting the co-expression of FVII and the endoprotease whereupon FVII is recovered from the culture medium. The medium used to culture the mammalian cells may be any medium suitable for growing mammalian cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). FVII produced by the cells may then be recovered from the cultur medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

Separation of the expressed product from the cell culture may be achieved by any method known in the art, including, without limitation, removal of cell culture medium containing the desired product from an adherent cell culture; centrifugation or filtration to remove non-adherent cells; and the like.

Purification of the crude product may be achieved using any method known in the art, including, without limitation, affinity chromatography. In the case of Factor VII or FVII related products one or more of the following methods may be used: an anti-Factor VII antibody column (see, e.g., Wakabayashi et al., *J. Biol. Chem.* 261:11097, 1986; and Thim et al., *Biochem.* 27:7785, 1988); hydrophobic interaction chromatography; ion-exchange chromatography; size exclusion chromatography; electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction and the like. See, generally, Scopes, *Protein Purification*, Springer-Verlag, New York, 1982; and

Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989. Following purification, the preparation preferably contains less than about 10% by weight, more preferably less than about 5% and most preferably less than about 1%, of non-Factor VII proteins derived from the host cell.

Factor VII and Factor VII-related polypeptides may be activated by proteolytic cleavage, using Factor XIIa or other proteases having trypsin-like specificity, such as, e.g., Factor IXa, kallikrein, Factor Xa, and thrombin. See, e.g., Osterud et al., *Biochem.* 11:2853 (1972); Thomas, U.S. Patent No. 4,456,591; and Hedner et al., *J. Clin. Invest.* 71:1836 (1983). Alternatively, Factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q® (Pharmacia) or the like. The resulting activated Factor VII may then be formulated and administered as described below.

The Factor VII or FVII related polypeptides produced by the present invention may be used to treat any Factor VII-responsive syndrome, such as, e.g., bleeding disorders, including, without limitation, those caused by clotting factor deficiencies (e.g., haemophilia A and B or deficiency of coagulation factors XI or VII); by thrombocytopenia or von Willebrand's disease, or by clotting factor inhibitors, or excessive bleeding from any cause. The preparations may also be administered to patients in association with surgery or other trauma or to patients receiving anticoagulant therapy.

Preparations comprising Factor VII-related polypeptides produced by the method according to the present invention, which have substantially reduced bioactivity relative to wild-type Factor VII, may be used as anticoagulants, such as, e.g., in patients undergoing angioplasty or other surgical procedures that may increase the risk of thrombosis or occlusion of blood vessels as occurs, e.g., in restenosis. Other medical indications for which anticoagulants are prescribed include, without limitation, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, myocardial infarction; Acute Respiratory Distress Syndrome (ARDS), Systemic Inflammatory Response Syndrome (SIRS), Hemolytic Uremic Syndrome (HUS), MOF, and TTP.

Pharmaceutical compositions comprising the Factor VII and Factor VII-related preparations produced according to the present invention are primarily intended for parenteral administration for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly. They may be administered by continuous or pulsatile infusion.

The following xamples are intended as non-limiting illustrations of the present invention.

Example 1: Cloning and characterization of SEQ ID NO:1, LRCA2

Subtractive PCR techniques have been used to identify genes which are expressed to high levels in Chinese Hamster (*Cricetulus griseus*) Ovary cells (CHO cells) (Puck, T. T, et al., 1958, "Genetics of somatic mammalian cells, III. Long-term cultivation of euploid cells from human and animal subjects". J. Exp. Med. 108:945-956; Kao F. T. and Puck T. T., 1968, "Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells". Proc. Natl. Acad. Sci. USA 60:1275-1281). One of the PCR products obtained by this assay, a 2.7 kb fragment was cloned from *Eco*RV digested CHO DNA (CHO cell line DG44) by use of DNA oligonucleotides CLC394 (AAAACTGGGAACCATTTGTG, SEQ ID NO:9) and CLC56L (CTGCAGAAGAGGCGACAG SEQ ID NO:10) and the PCR-Select kit (CLONTECH). CLC394L and CLC56L are complementary to the CHO cyclophilin cDNA sequence (Genbank Accession no. X17105).

Subsequent analysis of the flanking sequences led to the isolation of a DNA fragment from the upstream region of the CHO cell cyclophilin gene (Bergsma, D. J. and Sylvester, D., 1990, "A Chinese hamster ovary cyclophilin cDNA sequence". Nucl. Acids Res. 18:200) promoter. This DNA fragment has been identified by computer-analysis (Singh, G. B., Kramer, J. A. and Krawetz, S. A., 1997, "Mathematical model to predict regions of chromatin attachment to the nuclear matrix". Nucl. Acids Res. 25:1419-1425) as a putative S/MAR element, which was named LRCA2, SEQ ID NO:1.

Example 2: Cloning and characterization of SEQ ID NO: 2, B4B1

A recombinant Baby Hamster Kidney cell line expressing a recombinant protein was analyzed for the chromosomal DNA encompassing the integrated plasmid DNA. To this end, chromosomal DNA of the recombinant BHK cell line has been isolated by standard methods. The isolated DNA became subsequently subject of restriction enzyme digest. The restriction enzymes used were characterized as enzymes that did not digest sequences present within the integrated plasmid DNA. Such a restriction digest will give raise to DNA fragments containing all of the integrated plasmid and both up- and downstream flanking chromosomal DNA. Following digest, the generated DNA fragments were subjected to a ligation reaction, aiming at intra-molecular ligation giving raise to circular DNA molecules. These circular fragments were then transfected into bacteria, which subsequently were grown under antibiotic selection pressure. Only bacteria that have been transfected with DNA fragments containing

the integrated plasmid DNA, and therefore containing an antibiotic resistance gene, w re growing under these circumstances. Analysis of clones resulting from digestion with distinct restriction enzymes revealed that digestion with restriction enzyme *Hind*III resulted in the largest DNA fragments with about 3,500 base pairs up- and about 3000 base pairs downstream of the integration site. These DNA fragments have been isolated, sequenced and analyzed. The upstream sequence has been identified by computer-analysis (Singh, G. B., Kramer, J. A. and Krawetz, S. A., 1997, "Mathematical model to predict regions of chromatin attachment to the nuclear matrix". Nucl. Acids Res. 25:1419-1425) as constituting a putative S/MAR element, which was named B4B1, SEQ ID NO:2.

Example 3: Construction of reporter gene vectors for DNA transfection

In order to analyze the effect of the S/MAR elements LRCA2 and B4B1 on protein expression yields and expression stability, a series of plasmids has been constructed. These plasmids express a reporter gene Secreted Alkaline Phosphatase (SEAP), from an expression cassette that is or is not flanked by S/MAR elements. These constructs have been cloned as follows:

The plasmid pSEAP2-Hygro-MCS (Figure 1) was made by cloning of the Hygromycin B resistance gene from pIND(SP1)/Hygro (Invitrogen) as a 1.8 kb *Ase*I (blunt, fill in) fragment in the unique *Sal*I site (blunt, fill in) of pSEAP2-Basic (CLONTECH) and incorporation of a DNA MCS-linker GCTGGGCCCGATATCACCGGTTAATTAACTAGTTTAAAC SEQ ID NO:6 in to the unique *Eco*47III site.

To create plasmid pSEAP2-CMVp (Figure 2) primer JO-252 TATTAAGATCTAGTTATTAATAGTAATCAATTAC SEQ ID NO:7 and primer JO-253 TATATAAGCTTGATCTGACGGTTCACTAAAC SEQ ID NO:8 were used to generate a 0.6 kb PCR amplified DNA fragment containing the human CMV IE promoter. The fragment was cut with *Bgl*II and *Hind*III before cloning into pSEAP2-Hygro-MCS.

To create plasmid pB4B1-SEAP2-B4B1 (Figure 3) pB4B1-SEAP2 and pSEAP2-B4B1 were first made by inserting a 3.6 kb B4B1 *Bam*HI-*Hind*III (blunt, fill in) fragment at two locations in the pSEAP2-CMVp vector, into the *Mlu*I site (blunt, fill in) or into the *Eco*RV site which generated pB4B1-SEAP2 and pSEAP2-B4B1, respectively. pB4B1-SEAP2 and pSEAP2-B4B1 were cut with *Hind*III and *Age*I and the B4B1 containing DNA fragments from these digests were ligated to generate pB4B1-SEAP2-B4B1.

Plasmid pSEAP2-LRCA2 was made by inserting a 2.0 kb LRCA2 *Hin*dIII-*Eco*RI (blunt, fill in) fragment (SEQ ID NO:3) into the *Eco*RV site of pSEAP2-CMVp. pLRCA2-SEAP2 was made by cloning of a 2.3 kb LRCA2 *Mlu*I-*Bcl*I fragment (SEQ ID NO:4) in to the *Mlu*I-*Bgl*II sites of pSEAP2-CMVp. pLRCA2-SEAP2 and pSEAP2-LRCA2 were cut with *Bsu*36I and *Age*I and the LRCA2 containing DNA fragments from these digests were ligated to generate pLRCA2-SEAP2-LRCA2 (Figure 4).

The *Mlu*I/*Sfu*I LRCA2-CMV fragment in pLRCA2-SEAP2-LRCA2 (Figure 4) was replaced with a PCR amplified 2.3 kb LRCA2 fragment containing *Mlu*I and *Sfu*I restriction sites at the 5´and 3´ends, respectively, generating construct ΔLRCA2-SEAP2-LRCA2 (Figure 5). The PCR amplified LRCA2 fragment named ΔLRCA2 contains a 12 bp deletion in the large GT-repeat (SEQ ID NO:5).

Example 4: Construction of Factor VII cDNA expression vectors for DNA transfection

In order to analyze the effect of the S/MAR elements B4B1 and LRCA2 on clone-to-clone variability, expression levels and stability when expressing therapeutic proteins, a series of plasmids has been constructed. The plasmids express a therapeutic protein coagulation factor VII (FVII), from an expression cassette that is or is not flanked by S/MAR elements. The plasmids were made as follows:

To create pFVII-CMVp (Figure 6), a 1.7 kb *Hin*dIII-*Sal*I (blunt, fill in) DNA fragment containing the human Factor VII gene and the SV40 3' UTR/polyA signal was ligated into the *Hin*dIII and *Mfe*I (blunt, fill in) sites of vector pSEAP2-CMVp and thereby replacing the SEAP gene.

Factor VII expression vector ΔLRCA2-FVII-HYG-LRCA2 (Figure 7) was made by cloning of a *Nhe*I containing linker sequence at the *Sfu*I site in ΔLRCA2-SEAP2-LRCA2 (Figure 5). The modified ΔLRCA2-SEAP2-LRCA2 vector was afterwards digested with *Apa*I and *Nhe*I and the 7.0 kb LRCA2 fragment ligated to the 4.4 kb *Nhe*I/*Apa*I fragment from vector pFVII-CMVp (Figure 6) thus generating vector ΔLRCA2-FVII-HYG-LRCA2 (Figure 7).

The CMV-SEAP-HYG *Hin*dIII/*Sal*I fragment from the pB4B1-SEAP2-B4B1 (Figure 3) construct was replaced with the 3.5 kb *Hin*dIII/*Sal*I fragment from the pFVII-CMVp (Figure 6) construct containing the CMV-FVII- HYG elements, generating the B4B1-FVII-HYG-B4B1 (Figure 8) construct.

CHO-K1 cells (ATCC CCL-61), cultured in growth medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 100 IU penicillin and streptomycin, non-essential amino acids, and 5 mg/l vitamin K1), were transfected using Fugene 6 transfection reagent as per

manufacturer's instructions (Roche, Basel, Switzerland). Stable pools of transfectants w re obtained by Hygromycin selection as per manufacturer's instructions (Invitrogen, Carlsbad, CA). FVII protein yields in the culture medium were determined by standard sandwich ELISA technique (Novo Nordisk), well known to persons skilled in the art.

From the pools of transfectants, a limiting dilution cloning was performed. Briefly, 2 cells pr. well were seeded in 96-well microtiter tissue culture plates in growth medium containing Hygromycin. Following incubation for 13 days medium was replaced, and 24 hr later medium was harvested for FVII ELISA analysis. All samples were compared for FVII expression yields.

Table 1 shows that flanking of the Factor VII expression construct by either B4B1 or LRCA2 leads to decreased variation in expression levels between different founder clones.

Table 1

	pCMV-FVII- HYG	pB4B1-FVII-HYG-B4B1	pΔLRCA2-FVII-HYG-LRCA2
% variation in FVII protein expression	221	141	115

Example 5: Transfection of mammalian cells and SEAP assay

Chinese Hamster Ovary (CHO) DG44 cells maintained in MEM Alpha medium (Invitrogen, Cat # 22571) supplemented with 5% heat inactivated fetal bovine serum (Invitrogen), 108 mg/L L-proline (Sigma), and penicillin (100 units/ml)/streptomycin (100 µg/ml) (Invitrogen) at 37°C and 5% CO_2 were transfected using the GeneJammer transfection agent (Stratagene) according to the manufactures instructions. Briefly, cells seeded in 6-well cell culture plates were approximately 40-50% confluent on the day of transfection and transfected with 2 µg of linearized plasmid DNA.

To generate hygromycin B resistant cell cultures, transfected cells was one day post transfection propagated in MEM Alpha growth medium containing 350 µg/ml hygromycin B (Roche). After 10 to 14 days of selection, where the cells were re-fed every 2nd or 3rd day with selective medium, hygromycin B resistant cells were confluent in the wells. From the day of 100% confluency and forward the cell cultures were split 1:12 to 1:30 every 3rd or 4th day. On day 29 post transfection cell cultures were split to growth medium without hygromycin. On day 32, 39, and 46 post transfection each cell culture was washed with PBS, trypzined and

seeded to approximately 30% confluency in 200 µl growth medium in five wells in a 96-well cell culture plate. After 24 hours of growth at 37°C and 5% CO_2 100 µl culture supernatant was withdrawn from each micro titer plate well and used for assaying Secreted Alkaline Phosphatase (SEAP) activity. In order to be able to measure specific SEAP production levels each well was assayed for relative cell numbers using Alamar Blue (BioSource International) cell proliferation assay according to the manufactures instructions.

SEAP levels were measured fluorometrically using 4-methylumbelliferyl as substrate. Briefly, the harvested medium was incubated at 65°C for 15 min to inactivate endogenous alkaline phosphatase and the medium was clarified by centrifugation at 4000 x *g* for 5 in at 4°C. 50 µl of culture supernatant and 2- to 10-fold diluted samples was pippeted into 96-well plates and development in fluorescence ($Ex_{360\ nm}$ and $Em_{450\ nm}$) was followed as a function of time in a FLUOstar Galaxy (BMG Labtechnologies, Germany) fluorescence microplate reader after addition of 225 µl of 4-methylumbelliferyl phosphate liquid substrate (Sigma).

On day 32, 39, and 46 post transfection stable transfected cell pools transfected with plasmid pSEAP2-Hygro-MCS, pSEAP2-CMVp, pB4B1-SEAP2-B4B1, or pLRCA2-SEAP2-LRCA2 were assayed for SEAP production. In figure 9 are shown the results from one such experiment showing that flanking of the SEAP expression construct by either B4B1 or LRCA2 leads to increased expression levels. Additionally it is seen that SEAP expression is stable over time.

Example 6: *In Vitro* Hydrolysis Assay

The following method can be used to assay Factor VIIa bioactivity. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288, Chromogenix, Sweden), at a final concentration of 1 mM, is added to Factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of a test and a reference Factor VIIa.

Example 7: *In Vitro* Proteolysis Assay

The following method can be used to assay Factor VIIa bioactivity. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X

(0.8 microM) in 100 µl 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by th addition of 50µl 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-*p*-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of a test and a reference Factor VIIa.

All patents, patent applications, and literature references referred to herein are hereby incorporated by reference in their entirety.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

Sequenc s listing

SEQ ID NO:1

LRCA2 full s quence (2,715 bp):

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SEQ ID NO:3

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SEQ ID NO:4

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721   TTTTATGAAC TTGGGTGACA TTGTGTTTGG TGCATAGACA TTAAGAATTG CAATGTCCTC
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1681  AAGGTTAGGA AATTTTTCTT TTTTGGTTTT CTTGAAAATA TTTTCCCTGC TTTTGACCTG
1741  CCTTCTTCCC CTTCCTCTAT TCCTTTGGTT TTTGCATAGT GTCTCTGGCT TCCTGGATGT
1801  TTTATGCCTG GATTATTTTA GACTTAACAT TTTCTTTGAC CAAGGTATCC ATTTCTTCTA
1861  TCTTGTCTTC ACTGCCTGAG ATTCTCTCTT CTATCTCTTG TATTCTGTCA GTGAGGCTTG
1921  TCTCTGAGGT TCCTGTTGGG TTCTTAATTT TTTCATTTCC AGATTTCCTT CAGTTTGGGT
1981  TTTGTTTATT AATTCTATTT CCACTTTCAG GTCCTGAAAT GTTTTACTCA TTTTCCTCCC
2041  AGTATTTACA TTTTCATAGG TTTCTTTAAT GGATTTATTC ATTTCCTCTT CAAGGACCTT
2101  TTATGAATTC ATAAAATGTA TGTTAAGGTC CTTGCCTTGT GCTTCAGCTA TGTTGCATTC
2161  TCAGGGCCTA TTGTAATAGG GTTTTAGCAG GGACATATTG TCCTGGTTGT TATTGTCTGT
2221  GTTTTTGCTT TGGCATATAG ACGGCTGAGT TTGGGATGAT TGTAATTCTA GGTGCTGAT
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SEQ ID NO: 5

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721   TTTTATGAAC TTGGGTGACA TTGTGTTTGG TGCATAGACA TTAAGAATTG CAATGTCCTC
781   TTGGTGGATT TTCCTTTGAT GCCTATGTAG TATTCTTCCC AATCTCATCT GCTTAGTTTT
841   GGGTTTAAGT CTATTAGTCA GATATTAAAA TGACTGTATC GGCTTGCTTC TTAGGGCCAT
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1921   CTGTTGGGTT CTTAATTTTT TCATTTCCAG ATTTCCTTCA GTTTGGGTTT TGTTTATTAA
1981   TTCTATTTCC ACTTTCAGGT CCTGAAATGT TTTACTCATT TTCCTCCCAG TATTTACATT
2041   TTCATAGGTT TCTTTAATGG ATTTATTCAT TTCCTCTTCA AGGACCTTTT ATGAATTCAT
2101   AAAATGTATG TTAAGGTCCT TGCCTTGTGC TTCAGCTATG TTGCATTCTC AGGGCCTATT
2161   GTAATAGGGT TTTAGCAGGG ACATATTGTC CTGGTTGTTA TTGTCTGTGT TTTTGCTTTG
2221   GCATATAGAC GGCTGAGTTT GGGATGATTG TAATTCTAGG TGCTGAT
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SEQ ID NO:6

MCS-Linker

GCTGGGCCCGATATCACCGGTTAATTAACTAGTTTAAAC

SEQ ID NO:7

Primer JO_253

TATTAAGATCTAGTTATTAATAGTAATCAATTAC

SEQ ID NO:8

Primer JO-253

TATATAAGCTTGATCTGACGGTTCACTAAAC

SEQ ID NO:9

Primer CLC394

AAAACTGGGAACCATTTGTG

SEQ ID NO:10

Primer CLC65L

CTGCAGAAGAGGCGACAG

Claims:

1. A method for production of Factor VII polypeptides or Factor VII-related polypeptides comprising a) transfecting a mammalian cell with an expression vector comprising a nucleic acid molecule encoding Factor VII or a Factor VII-related polypeptide and expression control regions operatively linked to thereto and at least one S/MAR element; b) culturing the transfected cell under conditions for expression of Factor VII or a Factor VII-related polypeptide and c) isolating the expressed polypeptide by suitable means.

2 A method according to claim 1, wherein the expression vector comprises two S/MAR elements.

3. Method according to claim 1 or 2, wherein the S/MAR elements are selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, functional fragments thereof and sequences being at least 70% homologous thereto.

4. A method according to claim 2, wherein the S/MAR elements are the same.

5. A method according to claim 4, wherein the S/MAR elements are SEQ ID NO:1.

6. A method according to claim 4, wherein the S/MAR elements are SEQ ID NO:2.

7. A method according to claim 4, wherein the S/MAR elements are SEQ ID NO:3.

8. A method according to claim 4, wherein the S/MAR elements are SEQ ID NO:4.

9. A method according to claim 4, wherein the S/MAR elements are SEQ ID NO:5.

10. A method according to claim 2, wherein the S/MAR elements are SEQ ID NO:1 and 2, respectively.

11. A method according to claim 2, wherein the S/MAR elements are SEQ ID NO:2 and 3, respectively.

12. A method according to claim 2, wherein the S/MAR elements are SEQ ID NO:2 and 4, respectively.

13. A method according to claim 2, wherein the S/MAR elements are SEQ ID NO:2 and 5, respectively.

14. A method according to claim 1, wherein the S/MAR elements are located in a distance of from 0 to10 kb from the DNA encoding Factor VII or a Factor VII-related polypeptide and the expression control regions.

15. Method for production of polypeptides or proteins in transfected mammalian cells comprising a) transfecting a mammalian cell with an expression vector comprising a nucleic acid molecule encoding the desired polypeptide or protein expression control regions operatively linked to thereto and at least one S/MAR element selected from the group consisting of SEQ ID NO:1 or SEQ ID NO:2, functional fragments thereof and sequence being at least

70% homologous thereto; b) culturing the transfected cell under conditions for expression of the desired polypeptides or protein and c) isolating the expressed product by suitable means.

16 A method according to claim 15, wherein the expression vector comprises two S/MAR elements.

17. Method according to claim 15 or 16, wherein the S/MAR elements are selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, functional fragments thereof and sequences being at least 70% homologous thereto.

18. A method according to claim 18, wherein the S/MAR elements are the same.

19. A method according to claim 18, wherein the S/MAR elements are SEQ ID NO:1.

20. A method according to claim 18, wherein the S/MAR elements are SEQ ID NO:2.

21. A method according to claim 18, wherein the S/MAR elements are SEQ ID NO:3.

22. A method according to claim 18, wherein the S/MAR elements are SEQ ID NO:4.

23. A method according to claim 18, wherein the S/MAR elements are SEQ ID NO:5.

24. A method according to claim 16, wherein the S/MAR elements are SEQ ID NO:1 and 2, respectively.

24. A method according to claim 16, wherein the S/MAR elements are SEQ ID NO:2 and 3, respectively.

25. A method according to claim 16, wherein the S/MAR elements are SEQ ID NO:2 and 4, respectively.

26. A method according to claim 16, wherein the S/MAR elements are SEQ ID NO:2 and 5, respectively.

27. Isolated DNA sequence having the sequence SEQ ID NO:1.

28. Isolated DNA sequence having the sequence SEQ ID NO:2.

29. Isolated DNA sequence having the sequence SEQ ID NO:3.

30. Isolated DNA sequence having the sequence SEQ ID NO:4.

31. Isolated DNA sequence having the sequence SEQ ID NO:5.

32. Vector constructs comprising a nucleic acid molecule encoding Factor VII or a Factor VII-related polypeptide and expression control regions operatively linked to thereto and at least one S/MAR element

33. Vector construct according to claim 32, wherein the S/MAR elemens are selected from the group consisting of SEQ ID NO:1; 2; 3; 4 and 5.

34. Vector constructs comprising a nucleic acid molecule encoding a desired polypeptide or protein, expression control regions operatively linked to thereto and at least one S/MAR element selected from the group consisting of SEQ ID NO:1, 2, 3, 4 and 5.

35. Transfected mammalian cell lines comprising a vector construct according to claims 33 or 34.

Abstra t

The present invention relates to scaffold/matrix attachment regions isolated from hamster cells and their use for the industrial production of recombinant proteins. Thus the invention is related to a method for production of Factor VII polypeptides or Factor VII-related polypeptides comprising a) transfecting a mammalian cell with an expression vector comprising a nucleic acid molecule encoding Factor VII or a Factor VII-related polypeptide, expression control regions operatively linked to thereto and at least one S/MAR element; b) culturing the transfected cell under conditions for expression of Factor VII or a Factor VII-related polypeptide and c) isolating the expressed polypeptide by suitable means.

Figure 1

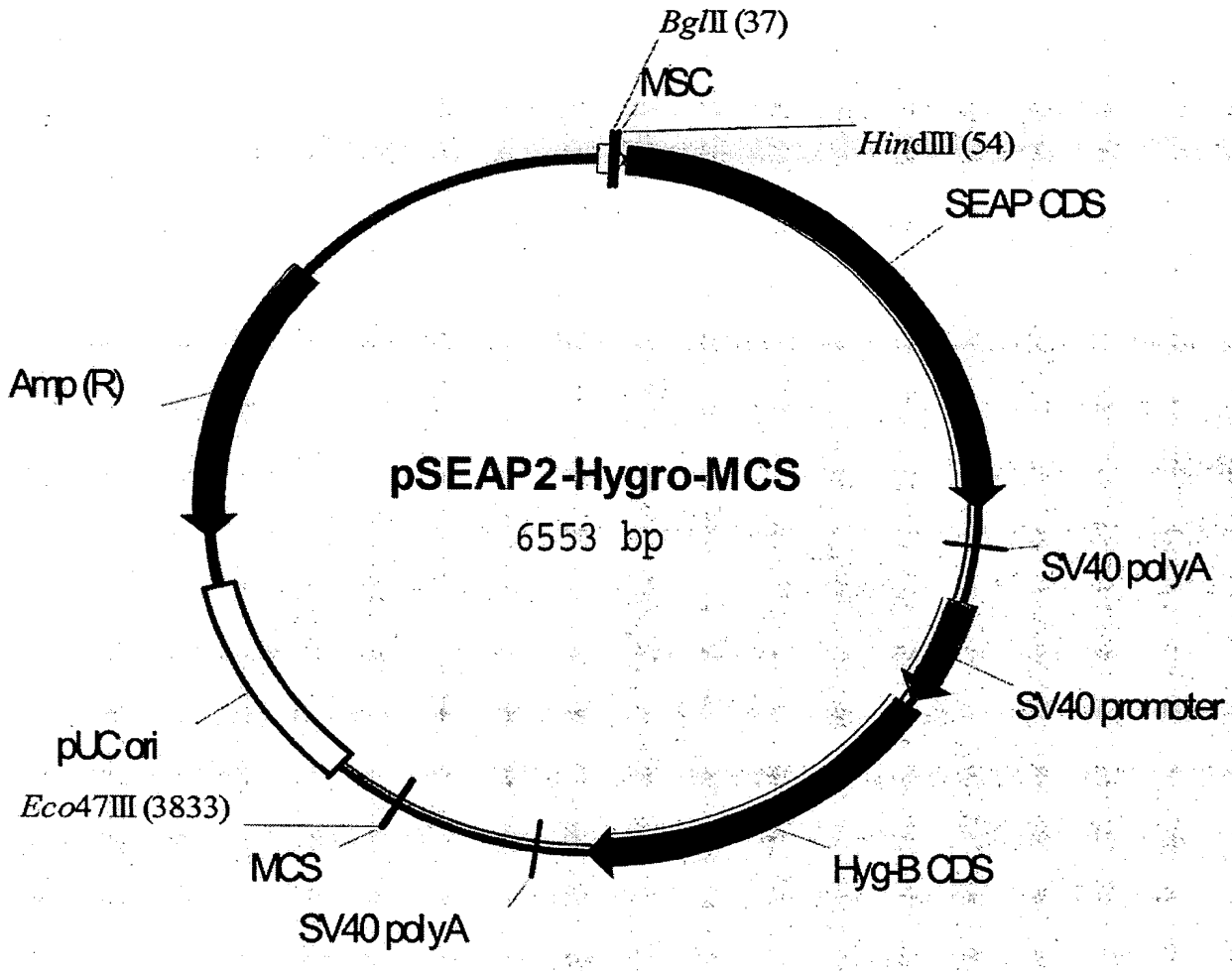

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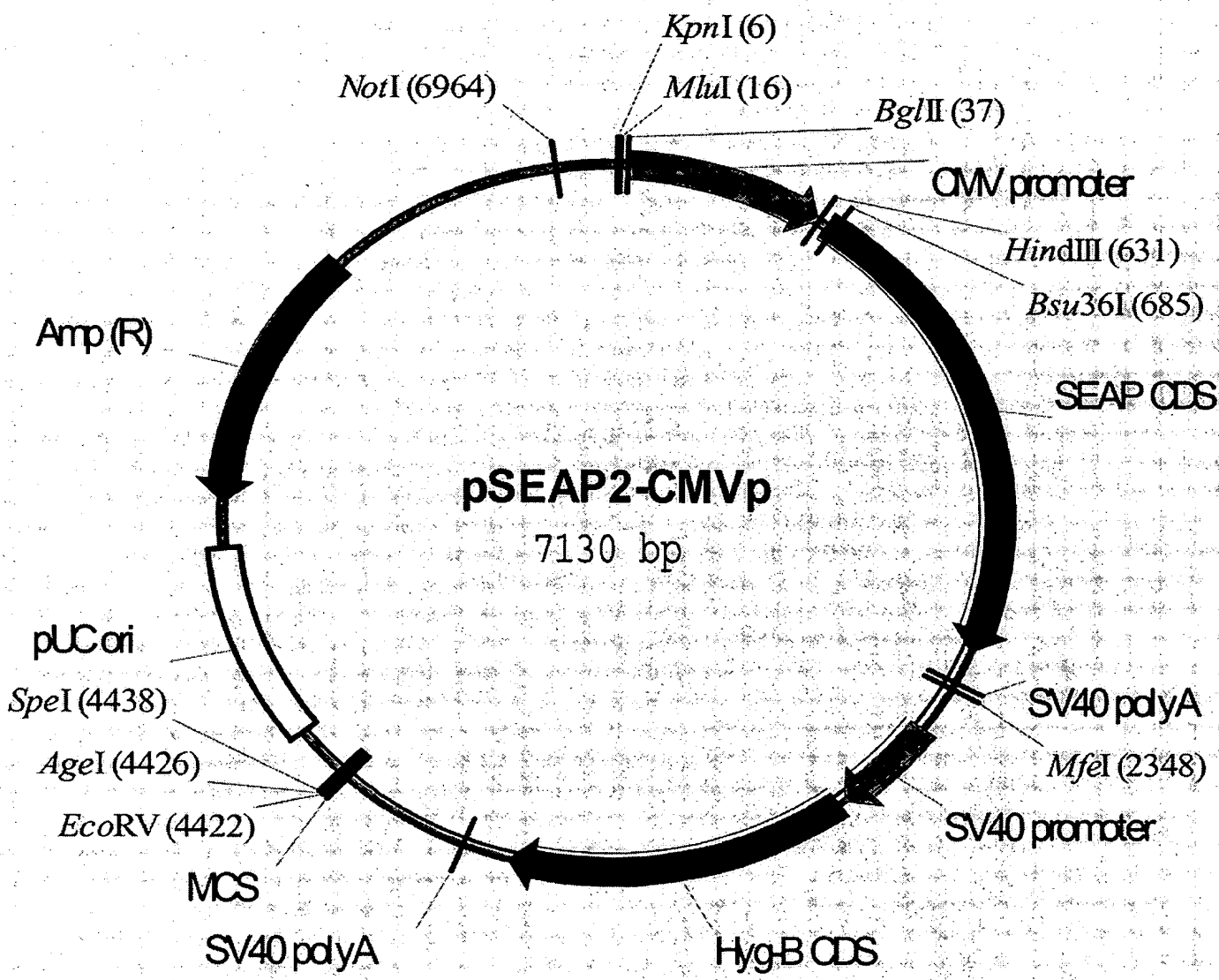
Figure 2



Figur 3

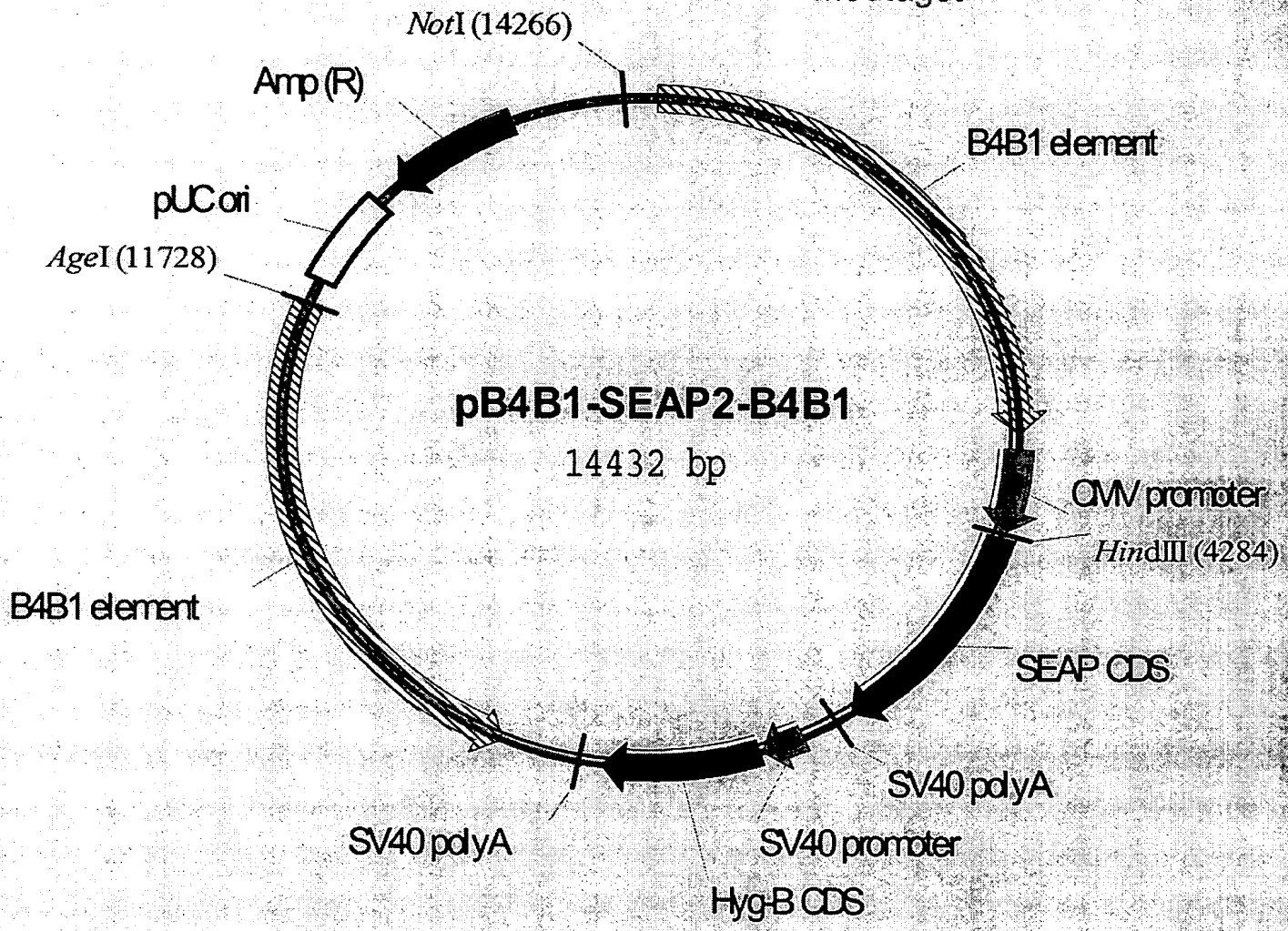


Figure 4

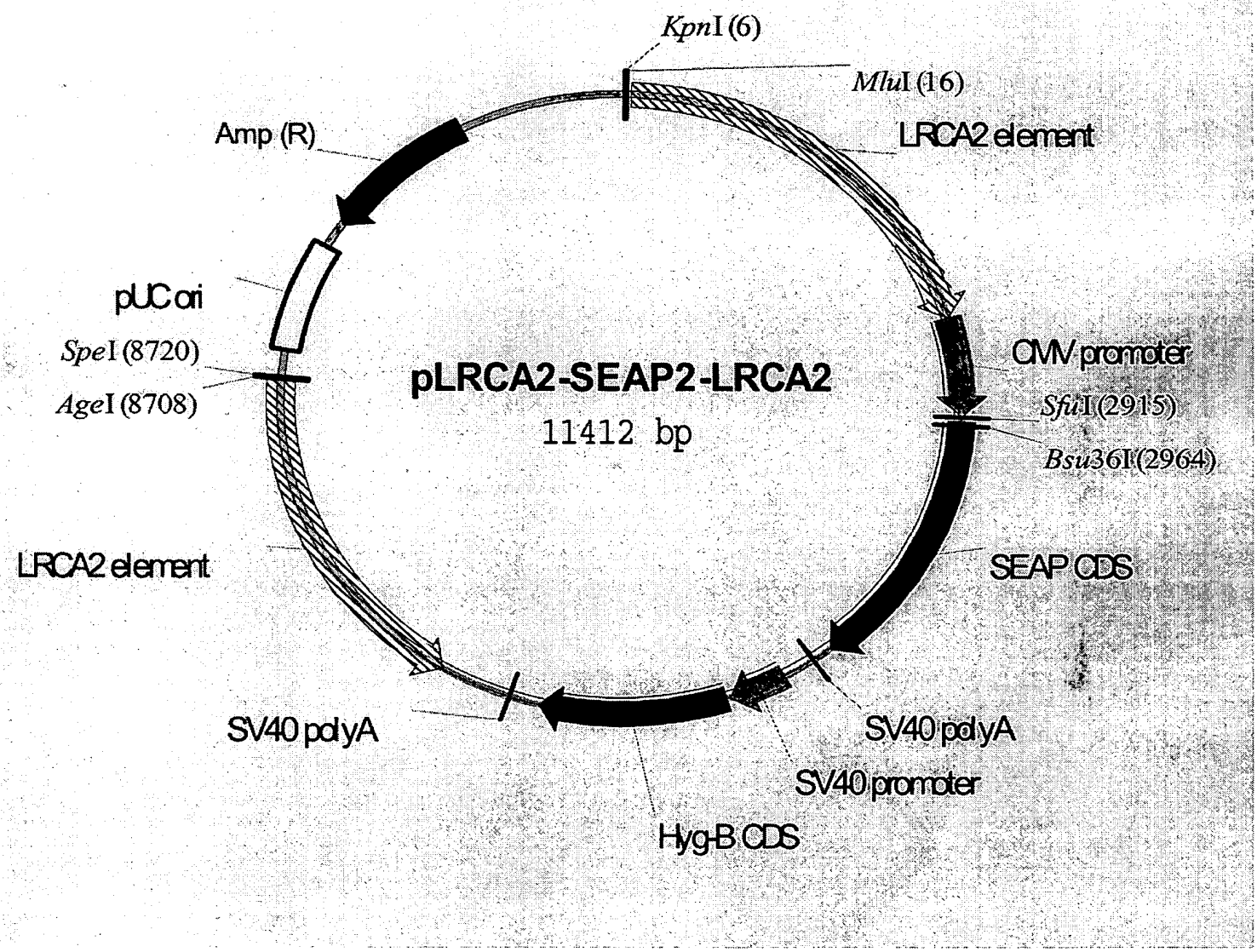


Figure 5

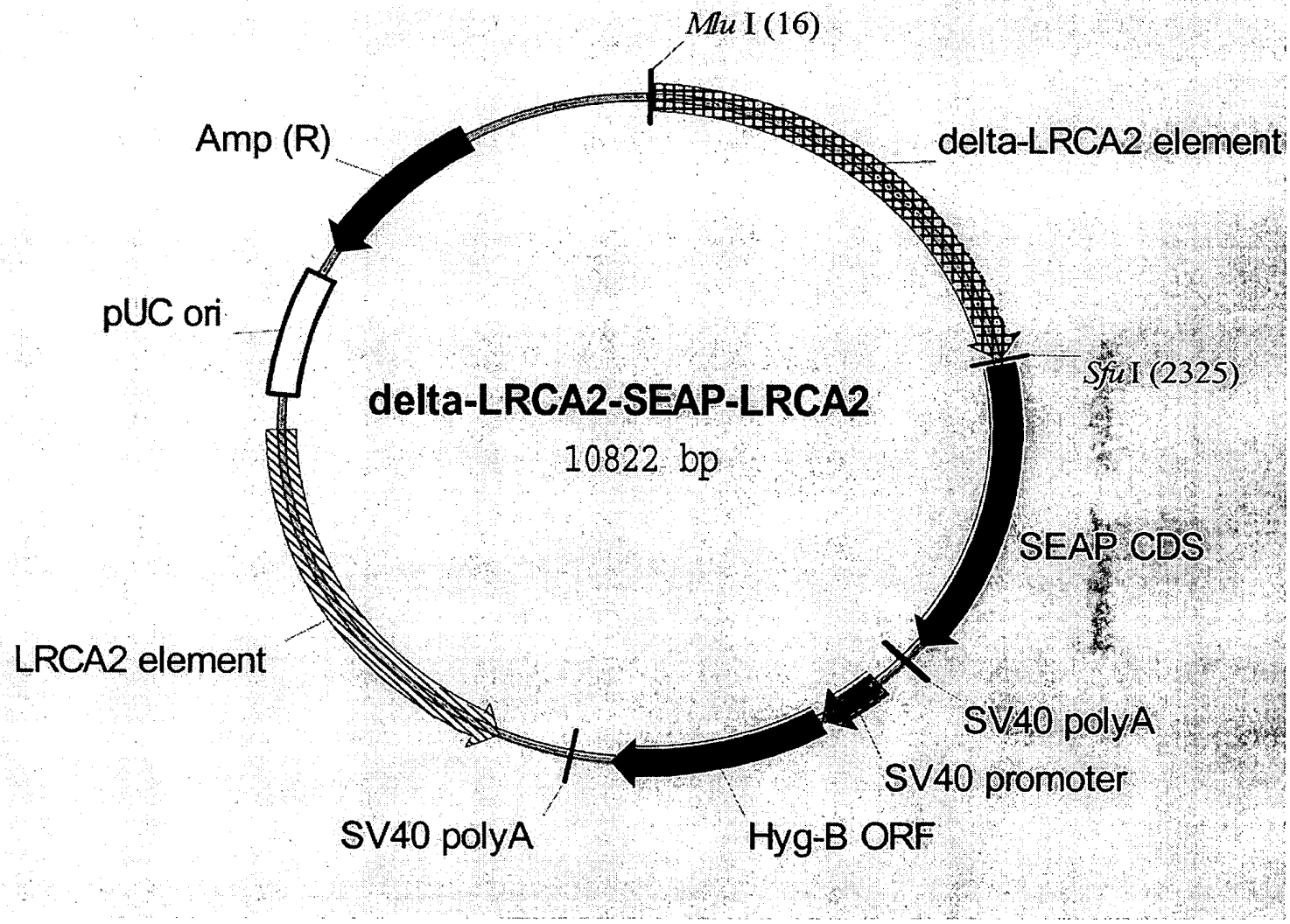


Figure 6

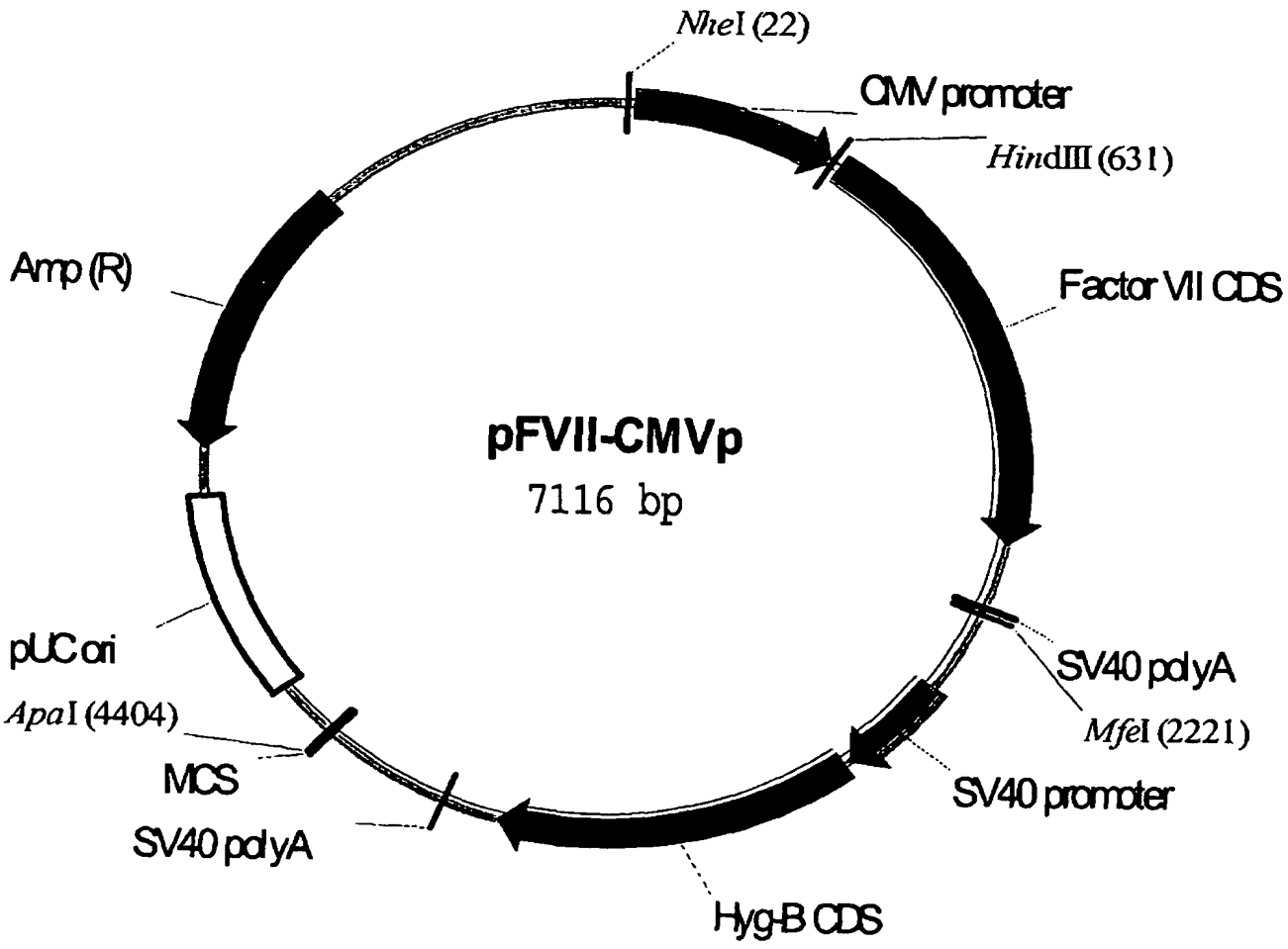


Figure 7

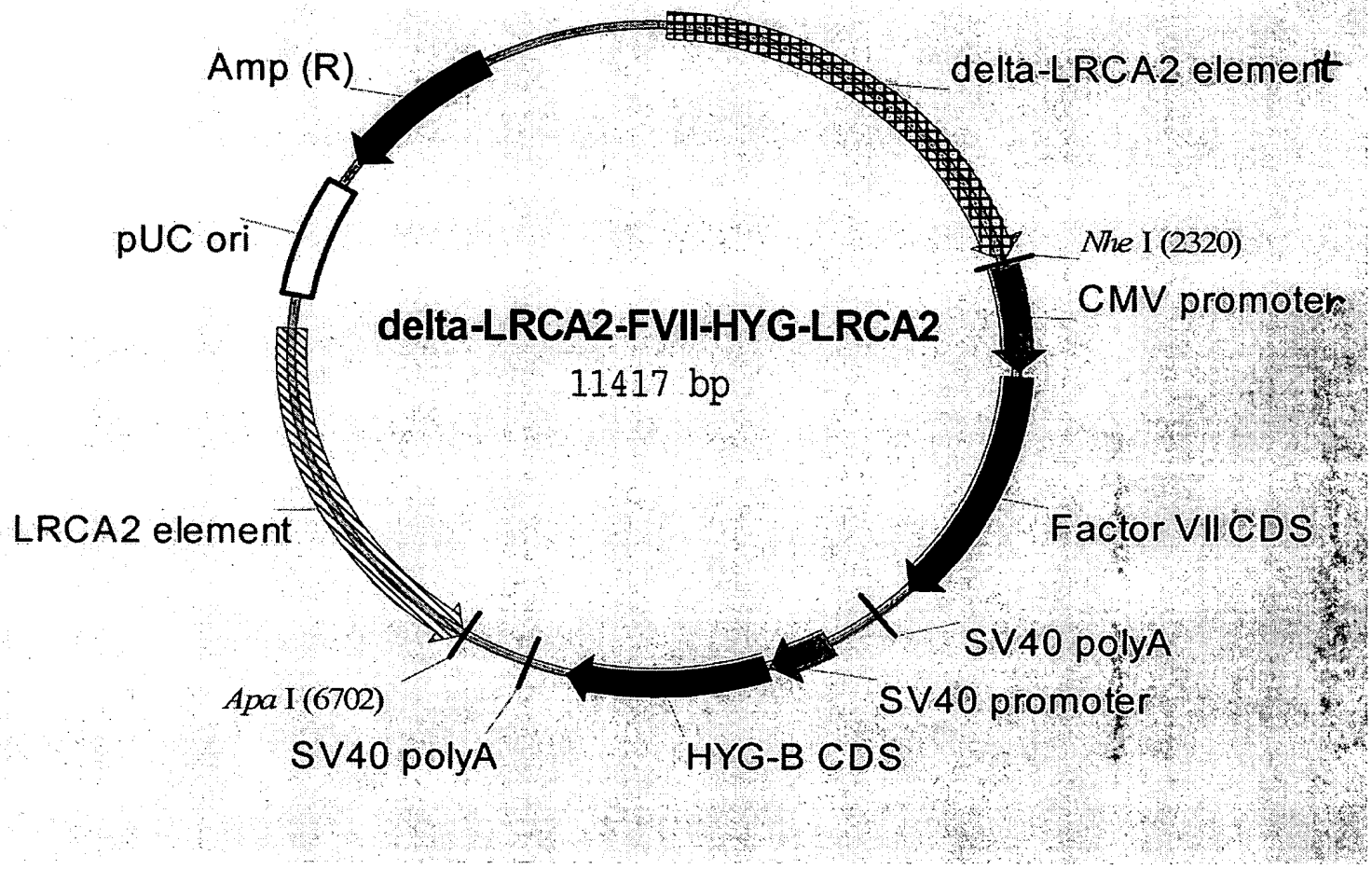


Figure 8

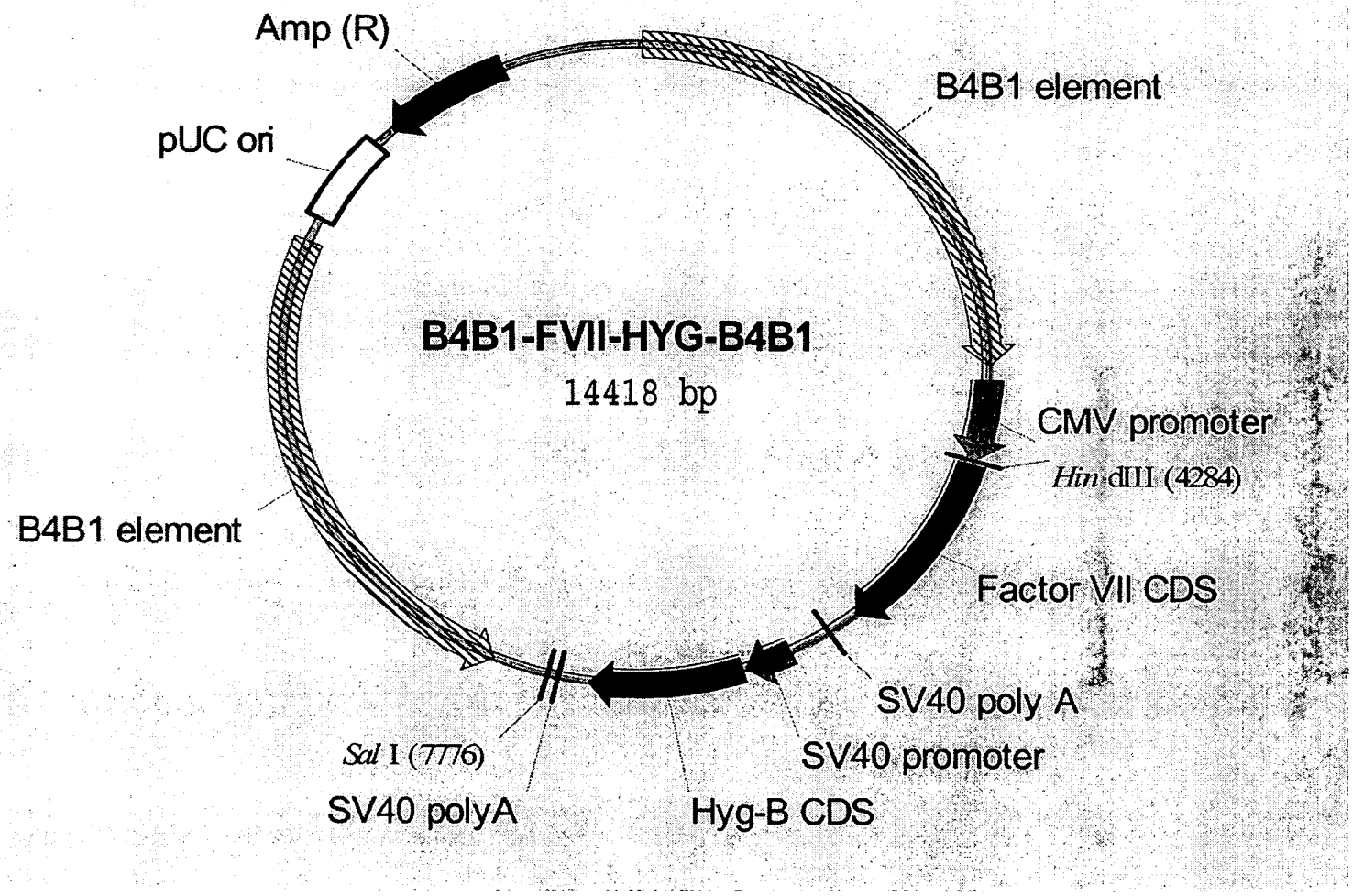


Figure 9